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Carcinoplacental isoenzymes; Ultrastructural studies in ovarian cancer and Regan and non-Regan isoenzyme producing HeLa cell lines

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SUMMARY

The carcinoplacental isoenzymes represent a sub-group of carcinoplacental proteins. Along with carcinofetal and carcinofetal-placental proteins these three sub-groups belong under the term carcinoembryonic proteins. Accordingly, in this classification, it is possible to retain an anatomic basis of reference in terms of the whole process of development.

Ultrastructural studies on the sites of alkaline phosphatase in human ovarian cancer cells have revealed an apparent association of Regan isoenzyme with mitochondrial membranes and of non-Regan isoenzyme with cell-surface membranes. The origin of the mitochondrial enzyme is unknown. Interesting arrays of mitochondria enveloped partially or entirely by endoplasmic reticulum exhibited ribosomes on the membranes facing the mitochondria were observed. These arrays were indistinguishable from those observed in cytotrophoblastic cells of the placenta.

Two new sub-lines of HeLa cells, rich in alkaline phosphatase are described. One, TCRC-1, produces only Regan isoenzyme and the other, TCRC-2, manufactures only non-Regan isoenzyme. TCRC-1 is further distinguished from TCRC-2 by its synthesis of acidic isoferritins and β-glucuronidase and by its ability to undergo alkaline phosphatase induction by prednisolone. These cell lines are expected to facilitate systematic studies on carcinoembryonic (fetal and placental) proteins with particular emphasis on the control of placental gene expression of isoenzymes.

The first group of carcinoplacental isoenzymes to be investigated consists of placental type alkaline phosphatase isoenzymes in human cancer patients. Thus, the Regan isoenzyme was discovered in 1968 in a metas-

tatic squamous cancer of the lung on the basis of a combination of enzyme tests involving L-phenylalanine inhibition, heat inactivation, starch gel electrophoresis and neuraminidase treatment[1]. This placental isoenzyme bears the name of the first such patient, Mr. P. Regan[2].

At this point, some reference to terminology is in order. Actually, the carcinoplacental isoenzymes belong in a classification of *carcinoembryonic proteins*, a term which should replace the limited term, "fetal antigens". The latter was inadequate to deal with protein antigens of the embryo, whose appearance precedes in time those of the placenta and of the fetus. Similarly, there was no provision before for distinguishing between antigens which were present in either fetus or placenta but not in both. Yet antigens of both fetus and placenta are of embryonic ancestry, which makes the term "carcinoembryonic" appropriate as a generic term applicable to all the protein antigens described in this Symposium which have an association with cancer.

The operational classification now in use at the Tufts Cancer Research Center appears in Table 1.

Table 1. *Classification of carcinoembryonic proteins*

Carcinoembryonic proteins		
Embryonic antigens		
Carcinofetal	*Carcinofetoplacental*	*Carcinoplacental*
CEA (Gold antigen)	acid isoferritins	*isoenzymes*
α-fetoprotein	pyruvate kinase	Regan isoenzyme
fetal isozymes		Nagao isoenzyme
hexokinase		Regan variant-Warnock
aldolase		*polypeptide hormones*
		chorionic gonadotrophin
		placental lactogen
		other proteins
		plasminogen activators
		angiogenesis factor

Tumor antigens which are not carcinoembryonic would include so called TSTA (tumor specific transplantation antigen), T-antigens and a variety of weakly antigenic viral proteins.

Carcinoplacental isoenzymes

From 1968 to the present, three carcinoplacental isoenzymes of alkaline phosphatase have been recognized. All of these heat-stable, L-phenylalanine-sensitive isoenzymes react with rabbit antisera to placental alkaline

phosphatase but differ in their electrophoretic migration in starch gel and in their sensitivity to L-leucine and EDTA.

Thus, the Regan isoenzyme migrates to the position of the F-phenotype[3] of placental alkaline phosphatase, is not inhibited by L-leucine but is by EDTA ; the Nagao isoenzyme corresponds on starch gel electrophoresis to the diffuse rarely-found D-variant of placental alkaline phosphatase whereas the Regan variant of Warnock and Reisman[4,5] migrates to a pre-albumin position and is sensitive to L-leucine but not EDTA.

From our published work[3], it is reasonable to suggest that the tumor isoenzymes of alkaline phosphatase correspond to normal phenotypes of placental alkaline phosphatase.

Isoenzymes of the non-placental type are also encountered in human cancer[6~9] but it has not been established if these have a carcinoembryonic origin.

Ultrastructural studies on cancer cells with Regan and non-Regan isoenzymes

In the course of an extensive study of alkaline phosphatase isoenzymes in ovarian cancer cells present in abdominal ascites fluid, we were able to define three classes. In one, the cells' alkaline phosphatase was inhibited by L-phenylalanine but not by L-homoarginine, an indication of Regan isoenzyme. In the second, the reverse situation applied-inhibition by L-homoarginine but not L-phenylalanine (non-Regan isoenzyme) and in the third class, there was inhibition by both L-phenylalanine and L-homoarginine (mixed Regan and non-Regan isoenzymes).

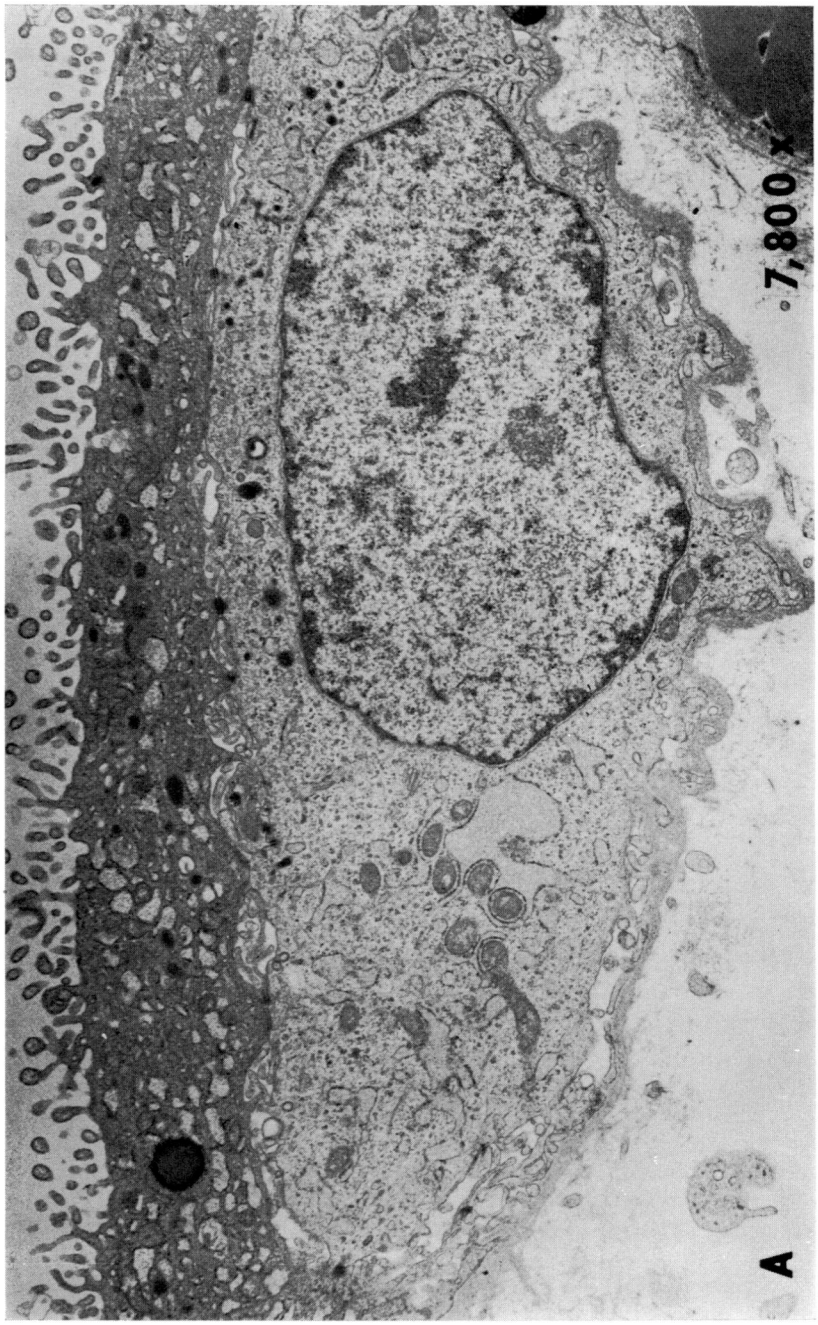
The details of this study are described in a paper soon to appear in *Cancer Research*[10] and so only the main findings are pointed out. here.

The ovarian cancer cells belonging to the first category-Regan type-exhibited deposits of enzyme reaction product in spaces between outer and inner mitochondrial membranes and occasionally cristae. An occasional deposit could be seen on the nuclear membrane and rough endoplasmic reticulum.

In the non-Regan cases, the most prominent sites were on the plasma membrane, and not the mitochondria.

It was interesting to note that cells exhibiting the mixed Regan and non-Regan isoenzymes showed a combination of alkaline phosphatase sites characteristic of the previous two cases mentioned.

It would thus appear that there may be a favored ultrastructural site for each of Regan and non-Regan isoenzymes in ovarian cancer cells. However, we would be surprised if this correlation held true in cancer cells of other types.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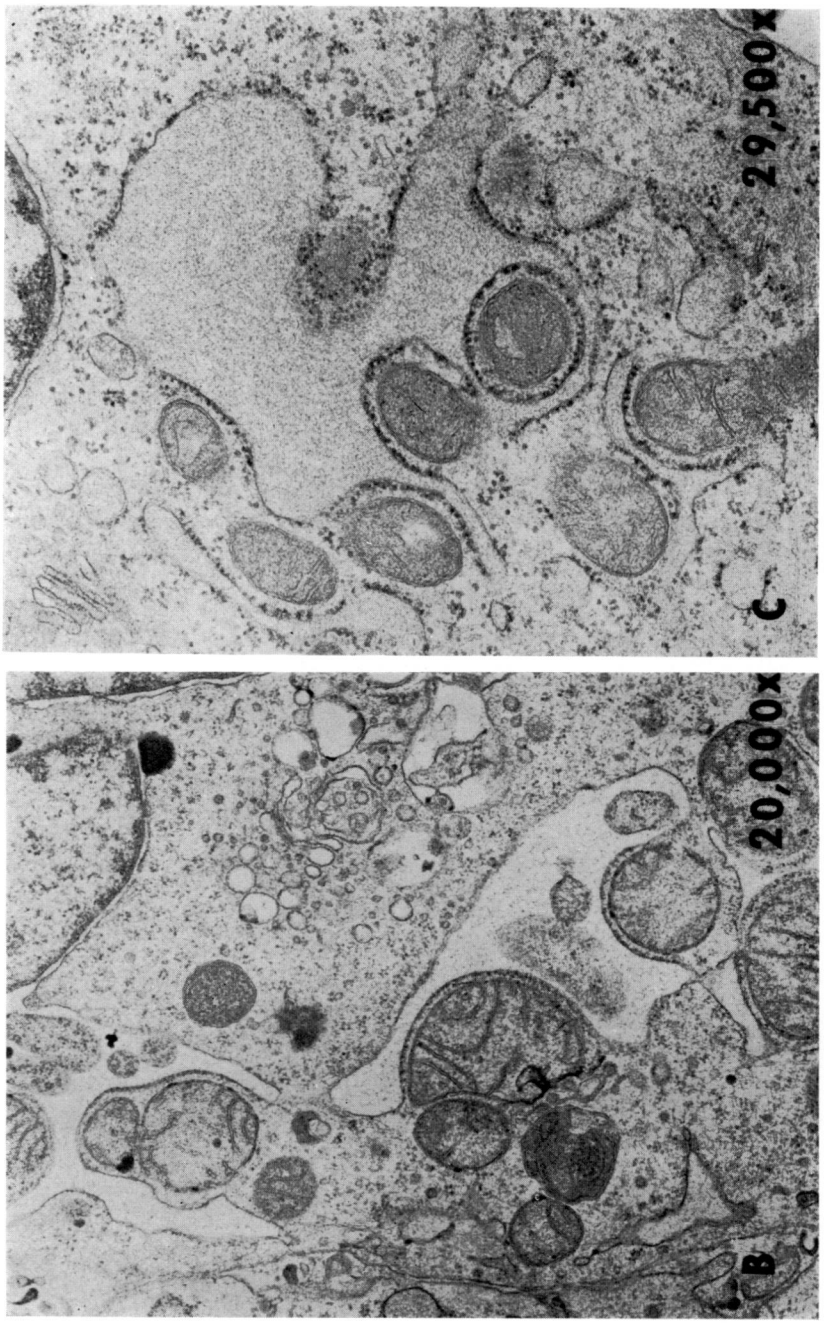

Fig. 1. A. Normal placenta: Cytotroph showing endoplasmic reticulum enveloping mitochondria.
B. Ovarian cancer cell with similar structures. Tissue shows alkaline phosphatase reaction.
C. Normal placenta. At higher magnification ribosomes can be seen facing mitochondria.

One finding in particular has intrigued us and this is being presented here more fully. This is the fact that we observed an unusual association of endoplasmic reticulum to mitochondria (case 2[10]); the mitochondria appeared to be partially and even wholly enveloped by profiles of endoplasmic reticulum whose ribosomes were oriented on the outer ER membrane facing the mitochondrion and sometimes two mitochondria (Fig. 1). We were struck with this picture because it matched the one(s) published by Dempsey[11] for cells of yolk sac epithelium of the cat and dog and in the cells of Langhans in human chorionic villi. No phosphatase staining was carried out by Dempsey.

Accordingly, we have studied chorionic villi of human placenta by electron microscopy and have confirmed the observations of Dempsey. Pictures of these endoplasmic reticulum-mitochondrial complexes are illustrated in Fig. 1.

This finding raises the question whether or not human cancer cells may express a placental morphology in part of their structure and perhaps for a period of finite duration. The function of these structures (in the placenta), Dempsey suggests, is to produce fetal serum especially during hemopoiesis and vasculogenesis and he relates them to the requirement of a combination of metabolic energy and protein synthesis.

That is ER-mitochondrial complex may occur in neoplastic cells other than human is clear from the recent study of Bruni on the similarity of fetal and neoplastic hepatocytes[12] and from the unpublished studies of Karasaki.

A model cell line producing a carcinoplacental isoenzyme

Several cell lines grown in culture have been found to produce placental type alkaline phosphatase.

HeLa cells show this property[2,13,14] as well as C-SPT cells[15].

We examined our wild-type HeLa cells and found that they were not only heterogeneous for placental alkaline phosphatase (some cells being strongly positive) as reported by others[16] but there were HeLa cells which were rich in non-Regan alkaline phosphatase. A successful attempt has now been made to clone out of wild-type HeLa cells Regan and non-Regan isoenzyme producing cells and to establish two cell lines TCRC-1 (Regan) and TCRC-2 (non-Regan).

In the following, I will describe our experiences so far in our studies of these two cell lines.

MATERIALS AND METHODS

Eagle's minimum essential medium with Earle's salts as modified for suspension culture, was supplemented with calf serum (final concentration 10%), streptomycin (100 μg/ml), penecillin (100 units/ml) and fungizone (2.5 μg/ml). After making this medium 0.1% with respect to sodium carbonate, the cells were grown in monolayer under standard conditions.

At each weekly passage, the monolayer of cells in each culture dish was incubated in 0.1% trypsin for 10 minutes, followed by the addition of an equal amount of growth medium and the transfer of the mixture to centrifuge tubes. After centrifugation for 5 minutes at 500 rpm, the pellet is resuspended in a measured volume of growth media and the cell population counted with a haemocytometer.

Viable cells (200×10^3), ascertained by trypan blue technique are suspended in 5 ml of growth media and are added to 30 ml Falcon tissue culture bottles. Media are changed every other day.

In order to select for HeLa cell clones exhibiting strong Regan and non-Regan isoenzyme activity, the technique of Mario and DeCarli[16] was used. Eventually the two desired cell lines were obtained. One, TCRC–1, produces only Regan isoenzyme as judged by inhibition by L-phenylalanine but not by L-homoarginine, heat-stability, and positive reaction with rabbit antisera to placental alkaline phosphatase. The other, TCRC–2, produces only non-Regan isoenzyme as shown by its inhibition by L-homoarginine but not L-phenylalanine, heat-lability at 65°, and a lack of reaction to rabbit antisera to placental alkaline phosphatase.

The activity of the alkaline phosphatase isoenzymes was determined by the hydrolysis of disodium phenylphosphate at pH 10.7[17] ; and the protein by the Lowry method[18]. The best conditions for measuring organ-specific amino acid inhibition were the ones employed. These are different than the ones (72 mM phenylphosphate pH 10.7) required for the placental alkaline phosphatase activity, before and after heating at 65° for 5 minutes.

Immunologic testing was carried out in two ways. For rapid detection, the cellulose acetate membrane technique of Inglis *et al.*[19] permitted identification of Regan isoenzyme in heated sera by virtue of its retarded migration in the presence of rabbit antisera. For precipitin line identification, the Ouchterlony double diffusion technique was used[20]. Visualization of phosphohydrolase activity is accomplished with 4.4 mM α-naphthyl phosphate substrate in 1 M 2-amino-2-methyl-1, 3-propanediol buffer, pH 9.68 which contains 0.233 mM $ZnSO_4$ $7H_2O$ and 3.0 mM $MgCl_2$ $6H_2O$, and 1.0 mg/ml of 4-amino-diphenylamine diazonium salt. The mixture is prepared

immediately prior to use, and is treated with activated charcoal and filtered.

The effect of corticosteroids (prednisolone, 1 μg/ml) were examined in cultures of 500×10^3 cells after one day in culture.

RESULTS

As shown in Table 1, the properties of HeLa TCRC–1 alkaline phosphatase activity match those of Regan isoenzyme with respect to L-phenylalanine inhibition and lack of inhibition by L-homoarginine, heat-stability and reaction to antisera to placental alkaline phosphatase.

Purified HeLa TCRC–1 alkaline phosphatase shared a line of identity without spur formation with highly purified placental alkaline phosphatase on Ouchterlony double-diffusion agarose plates whereas the non-Regan isoenzyme exhibited no precipitin band when tested against rabbit antisera to purified human placental alkaline phosphatase.

Studies of the chromosome number of both HeLa cell lines showed a difference. Thus, whereas the TCRC–1 Regan isoenzyme line has a chromosome number range from 59 to 71 peaking at 67, the TCRC–2 non-Regan line distribution ranges from 59 to 72 with a modal chromosome number of 64.

Contrasting results were observed in the two cell lines as a consequence of adding prednisolone to their cultures in that TCRC–1, the Regan isoenzyme line experienced an enhancement in activity whereas the TCRC–2 line did not (Table 2).

Table 2. *Some characteristics of Hela clones*

	TCRC–1	TCRC–2
Alkaline phosphatase		
in viro		
Specific activity (μmoles phenol/min/mg protein)	0.8	3.2
L-phenylalanine inhibition	73.1%	0
L-homoarginine inhibition	11.5%	77.5%
Heat inactivation (5 min at 65°C)	10.9%	100%
Reaction to placental Ab.	+	−
in culture		
Prednisolone effect (24 hrs) on specific activity	+175%	0%
β-glucuronidase (μg phenolphthalein/hr/mg protein)	32	5.8
Acidic isoferritins	+	−
Modal chromosome number	67–69	64

An electrophoretic comparison using the Beckman "microzone" apparatus and cellulose acetate membrane strips indicated that uninduced and prednisolone-induced alkaline phosphatase possessed the same migration. Moreover, both were placental in type because their migration was slowed down in the presence of rabbit antiserum to placental alkaline phosphatase.

In other experiments (summarized in Table 1) the cell lines were found to differ in β-glucuronidase activity and in isoferritins (Drysdale, Singer, Angellis and Fishman, unpublished observations).

DISCUSSION

The experimental evidence indicates that two sublines of wild type HeLa have been established; TCRC–1 is a producer of Regan isoenzyme-placental type alkaline phosphatase, whereas TCRC–2 manufactures non-Regan isoenzyme— the non-placental alkaline phosphatase. Other significant differences in content of acidic isoferritins and β-glucuronidase have also been described, as well as the ability of TCRC–1 but not TCRC–2 to undergo enhancement of its alkaline phosphatase by the presence of prednisolone in the culture medium.

The explanation for the phenomenon of placental alkaline phosphatase synthesis may be at the level of transcription, translation or of both these processes. A suggestion that the genome may be involved comes from the fact that the chromosome number differs in the two lines and that in the literature a correlation of karyotype with the expression of alkaline phosphatase has been noted[13,17].

Certainly the availability of these two cell lines makes feasible a systematic study of the control of gene expression of a carcinoplacental phenotype and of a non-placental phenotype both sharing the same catalytic activity. We are hoping to find additional carcinoplacental isoenzymes and these cell lines would make it possible to find if they are linked to the genes coding for Regan isoenzyme.

With regard to the observations on the ultrastructural sites of Regan and non-Regan isoenzymes in human ovarian cancer cells, we have noted what appears to be a degree of organelle specificity. The Regan isoenzyme appears to be associated principally with mitochondria and the non-Regan isoenzyme with cell surface membranes. Whether or not this correlation holds for all types of cancer is a question that can only be answered as a result of much more work.

What remains intriguing to us and a subject of current investigation is the significance of an interesting complex of endoplasmic reticulum and mitochondria in a number of ovarian cancer cells that is indistinguishable

from similar complexes in the cytotrophoblastic cells of human placental chorionic villi.

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